METHYLMERCURY-INDUCED DYSFUNCTION OF BLOOD VESSEL CELLS: IMPLICATIONS IN CARDIOVASCULAR DISEASES

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ABSTRACT

Methylmercury [MeHg] has long been known as a neurotoxic agent. Recent epidemiological research has also linked MeHg exposure to cardiovascular disease. However, the precise mechanisms linking MeHg toxicity to blood vessels at the cellular levels are lacking. Endothelial cells exist as a single monolayer of cells that form the inner lining of blood vessels and are crucial to the structure and function of blood vessels. It was proposed that these cells link MeHg cytotoxicity to cardiovascular disease. The aim of this study was therefore to observe the effects of MeHg on endothelial cells. This study revealed that increasing concentrations of MeHg had significant effects on cytotoxicity and loss of cell viability in endothelial cells. This is the first study offering a connection between the adverse effects of MeHg at the cellular level and the cardiovascular diseases due to MeHg exposure, confirming empirical evidence at this level.

1. INTRODUCTION

Elemental mercury has long been known as an environmental contaminant. Ever since the early 1960s, the clean air act and other EPA legislations have identified mercury as a dangerous air, water and food contaminant (Egermayer 2000). This is because human mercury poisoning has profound neurological effects for animals and humans. It has been shown to result in tremors, hallucinations, psychotic behavior, and delirium (McFarland 1978). Mercury also has been identified as one of the heavy metals that undergo biomethylation in the environment and become ten times as toxic as compared to inorganic mercury (Dopp et. al. 2004; Boening 2000). Through biomethylation, mercury turns into methylmercury [MeHg] through bacterial metabolism in the silt underwater. This compound often collects at the top of food chains through bioaccumulation of the toxin (Dopp et. al. 2004). Seafood especially constitutes a large source of MeHg in the daily diet has lead to concerns of neurological health hazards among seafood consumers (Landmark 2004; Balshaw 2007).

Studies on populations that have been exposed to MeHg have also been shown to disproportionately exhibit *cardiovascular* diseases, whether in the form of hypertension or cardiac infarction (Egermayer 2000; Kostka 1991). Higher levels of mercury, due to fish consumption, are seen as a risk factor in coronary heart disease (Yoshizawa et al. 2002). Increased levels of mercury in the blood have also been associated with elevated cholesterol levels in humans. In 2002, Guallar et al. reported that "the risk-factor—adjusted odds ratio for myocardial infarction associated with the highest as compared with the lowest quintile of mercury [exposure] was 2.16…high mercury content may diminish the cardioprotective effect of fish intake." Mercury has been suggested as a risk factor of myocardial infarction, coronary disease, and cardiovascular diseases (Kim et al. 2005).

Although epidemiological data have shown a link between vascular disease and mercury toxicity, there have been no cellular or molecular toxicity data to corroborate this connection. Endothelial cells form a single monolayer of cells that function as the inner lining of the blood vessels and are thus critical to the proper function of blood vessels and the circulatory system. It was proposed that the effects of MeHg on endothelial cells offered an explanation for the cardiovascular diseases due to MeHg exposure. The adverse effects of MeHg were thus studied on a widely accepted and established bovine lung vascular endothelial cell line. They were hypothesized to adversely affect the cell line.

This experimental model would then show whether MeHg exposure would have adverse effects on vascular endothelial cells, and thus be a cause of environmentally-induced cardiovascular disease.

2. MATERIALS AND METHODS

2.1 Supplies

The type of vascular endothelial cell culture used was BPAECs [Bovine Pulmonary Artery Endothelial Cells] from American Type Culture Collection Cell Lines (San Diego, CA). They were grown in MEM [Minimum Essential Medium, Sigma Aldrich Serial Number (SASN): M4526], which consisted of 10% fetal bovine serum and a 2% antibiotic cocktail. Methylmercury [MeHg], the chemical tested for its cellular effects, was also purchased from Sigma-

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Aldrich (SASN: 33368) at 99% concentration. Lactic Dehydrogenase [LDH] Assay kits, necessary for analyzing the effects of MeHg were also bought from Sigma-Aldrich (SASN: TOX7-1KT). A chemical necessary for this analysis but not present in the assay kit was 1-M HCl (SASN: H1758). Finally, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (abbreviated as MTT) assay kits were procured from Molecular Probes (serial number: V13154). Dimethyl Sulfoxide [DMSO] (SASN: D2650) was, again, necessary for this analysis but not present in the kit.

2.2 Apparatus

All cells were grown in 24 well plates, where each cell has a volume of 250 µL. Plastic, conical eppendorf tubes were used for LDH analysis after 24-well growth. Both LDH and MTT assays necessitated transfer of cell supernatant solutions to 96 well micro plates. The majority of the analyses done for this lab used a spectrophotometer to assess the level or mitochondrial or cell membrane viability in a particular test sample. In this case, the Soft Pro Max UV-Vision Instrument was used for such data collection. Another important tool for final data collection was the Olympus Eclipse TE2000-S, an invertible research microscope. Images were captured digitally from the system at 40X magnification.

2.3 Procedure

BPAECs were cultured in MEM at 37°C in an incubator consisting of 5% CO_2-95% air. After approximately 24 hours of growth, cell cultures were checked for 75-90% confluence and typical cobblestone morphology under the microscope. All cell cultures that met these criteria continued on to cytotoxicity trials.

Out of these eligible batches, a single 4x6 *cell system* was chosen for continued experimentation. Therefore, 24 cell plates were chosen. Every 3 cell plates needed to undergo the same treatment for confirmation of data, so 8 different cytotoxicity trials emerged. In these trials, cell lines were exposed to different concentrations of MeHg, as well as exposed to a single concentration of MeHg for differing durations of time. These trials are summarized in table 1 and schematically demonstrated in figure 1.

Time-Based Trials (concentration of MeHg [5 µM] constant)	Dose-Based Trials (duration of MeHg exposure [60 min] constant)
Control [No Exposure]	Control [No exposure]
15 minutes of exposure	1 µM of exposure
30 minutes of exposure	5 µM of exposure
60 minutes of exposure	10 µM of exposure

TABLE 1: TIME-BASED AND DOSE-BASED CYTOTOXICITY TRIALS

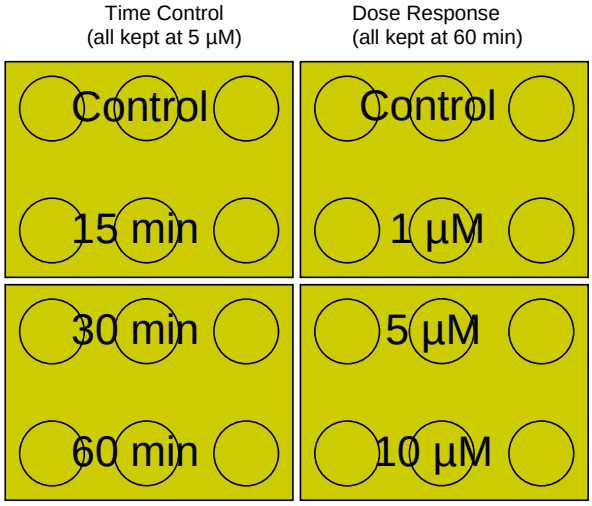

FIGURE 1: SCHEMATIC OF TIME-BASED/ DOSE-BASED TRIALS

After different MeHg treatments are delivered, three aspects of cells were measured to assess overall cellular viability:

1. cell membrane viability (through the LDH assay)
2. cell mitochondrial viability (through the MTT assay)
3. cell death (through cell morphology analysis)

The LDH assay of cell membrane viability is based on the fact that LDH is a large enzyme that is present in negligible quantities if the cell membrane is viable. Yet in affected cells, LDH escapes through a nonfunctional membrane into the surrounding supernatant. Therefore, the amount of LDH enzyme present in the supernatant is directly related to how permeable cell membranes in a certain cell line are.

To begin the LDH protocol, 125 µL of supernatant of a cell sample was placed into an Eppendorf tube. Afterwards, 2.5 mL of LDH cofactor (Lactate), 2.5 mL of LDH dye solution (a tetrazolium dye extract), and 2.5 mL LDH substrate (NAD^+) (all present in the LDH assay kit) was added into the tube. With the presence of Lactate, any LDH present allows NAD+ (Nicotinamide adenine dinucleotide) turn to NADH (see figure 2). This NADH, in turn, was utilized in the stoichiometric conversion of the tetrazolium dye present.

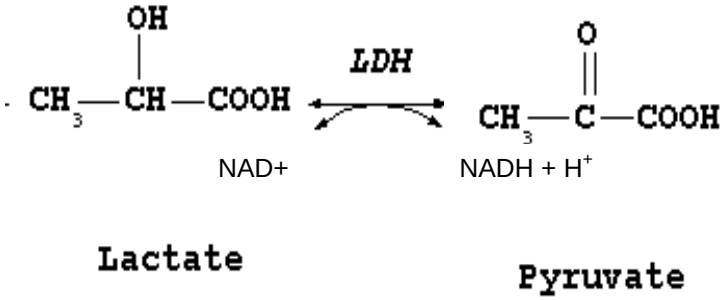

FIGURE 2: THE CONVERSION OF NAD^+ INTO NADH

After letting the samples sit for 20 minutes, 37.5 µL of 1 M HCl was added to the eppendorf tube to terminate this reaction. 100 µL of the resulting sample was put into one well in a 96-cell well plate. The result was read at 490 nm (according to manufacturer specifications) in the spectrophotometer. The greater the percent absorbance read (in other words, the greater the intensity of the colored solution), the greater the percent of LDH in the supernatant—and the greater the membrane permeability of the cell.

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, on the other hand, assesses the mitochondrial health of the cell samples. In this assay, the supernatant of a cell sample was aspirated and discarded. It was replaced with 250 µL of minimum essential medium [MEM], as well as 25 µL of a 12 mM MTT solution. If cells were viable, mitochondrial dehydrogenases cleaved the tetrazolium ring present in the sample by donating protons (see figure 3).

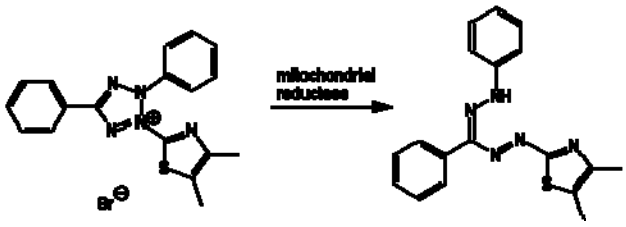

FIGURE 3: THE CLEAVING OF A DIPHENYLTETRAZOLIUM RING

After a 2 hour incubation period, insoluble purple crystals appeared to precipitate out of the supernatant. These crystals were actually the reduced product in figure 3. After 212.5 µL was removed from each well, 125 µL of dimethyl sulfoxide [DMSO] was added to dissolve these products back into the solution. 100 µL of the resulting supernatant was taken from each cell well and put into the 96-cell well plate to be read by the spectrophotometer at 540 nm. In this analysis, the greater the percent absorbance read (in other words, the greater the intensity of the color present), the greater the viability of mitochondria in these cells.

The final analysis of the cell cultures involved the viewing of samples under the Nikon dissecting microscope (magnification: 40X) to perform a cell death count. Cell samples were exposed to varying concentrations of MeHg (0 µM, 1 µM, 5 µM, and 10 µM) for 60 minutes. The results were seen under the microscope. Dead cells are identified as cells that have shriveled into a small sphere, while live cells were considered as ones that preserved a cobblestone morphology—a shape that is characteristic of normal mammalian endothelial cell layers.

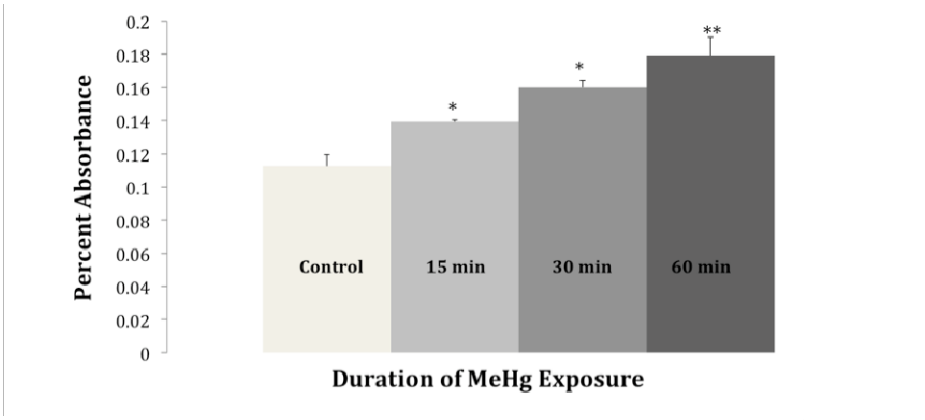

FIGURE 4: THE EFFECTS OF PROLONGED MeHg EXPOSURE ON ABSORBANCE (LDH ASSAY)

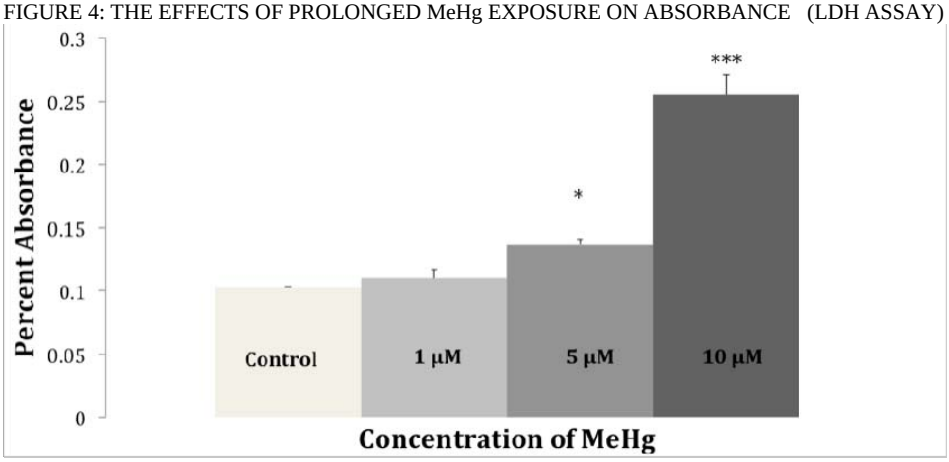

FIGURE 5: THE EFFECTS OF CONCENTRATION OF MeHg ON ABSORBANCE (LDH ASSAY)

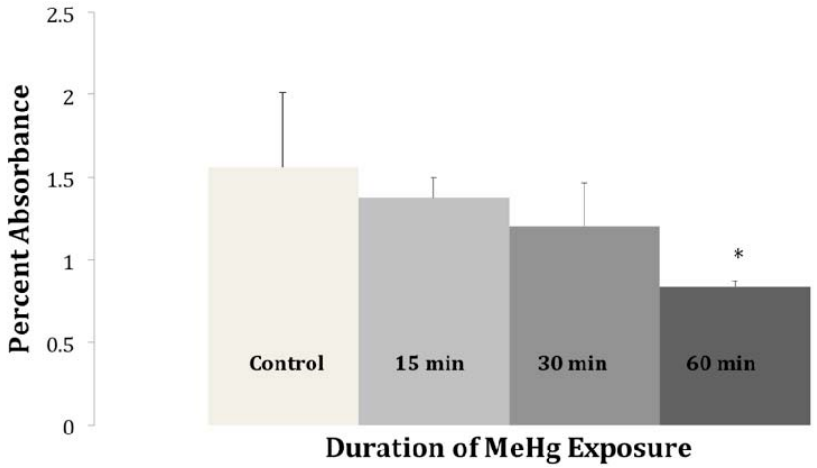

FIGURE 6: THE EFFECTS OF PROLONGED MeHg EXPOSURE ON ABSORBANCE (MTT ASSAY)

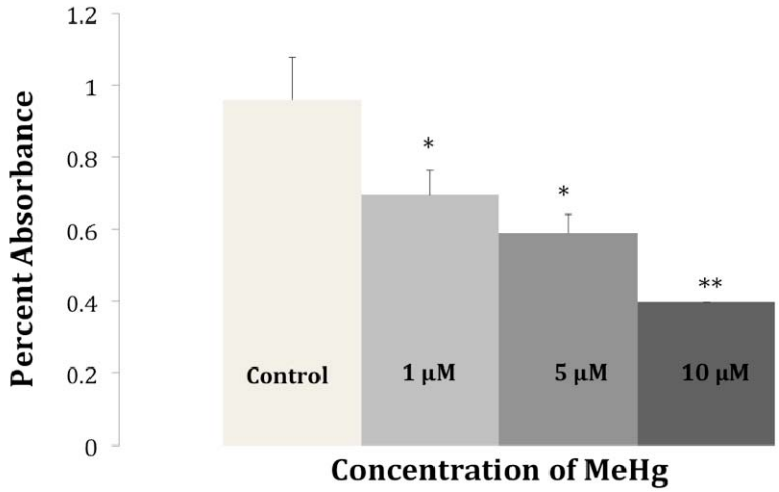

FIGURE 7: THE EFFECTS OF CONCENTRATION OF MeHg ON ABSORBANCE (MTT ASSAY)

3. Results

The LDH time and dose response trials showed strong positive correlations. As was expected, the percent absorbance (or the intensity of solution color) of both trials increased dramatically with greater durations of exposure to MeHg as well as with greater concentrations of MeHg. The dose response shows an exponential regression with little deviation in data points. Meanwhile, a much more gradual, linear regression is shown with the time response trials. This convincing evidence that the increase in concentration of MeHg and the increase in the duration of MeHg exposure both increase cell membrane permeability, allowing large molecules to leak through the membrane from the cell. This translates to loss of cell membrane functionality as levels and durations of MeHg exposure increase.

Meanwhile, the MTT data show a strong negative correlation with respect to both time and dose response trials. The decrease in percent absorbance in the MTT assay is a strong indication of the decline in cell mitochondrial function as concentrations and durations of MeHg exposure increase. It is important to note again

that dose-response trials show a much steeper and stronger negative correlation. Uncertainty values for this data set are tighter, and thus show statistical significance. The time-response trials, while still undoubtedly showing a negative correlation, have larger uncertainty values, which results in the 15 minute and 30 minute values overlapping in tolerance. This makes the data set only partially significant statistically.

Cell morphology pictures (taken at 40X magnification) also corroborated previous findings, showing higher levels of cell death with greater MeHg levels (see figure 8).

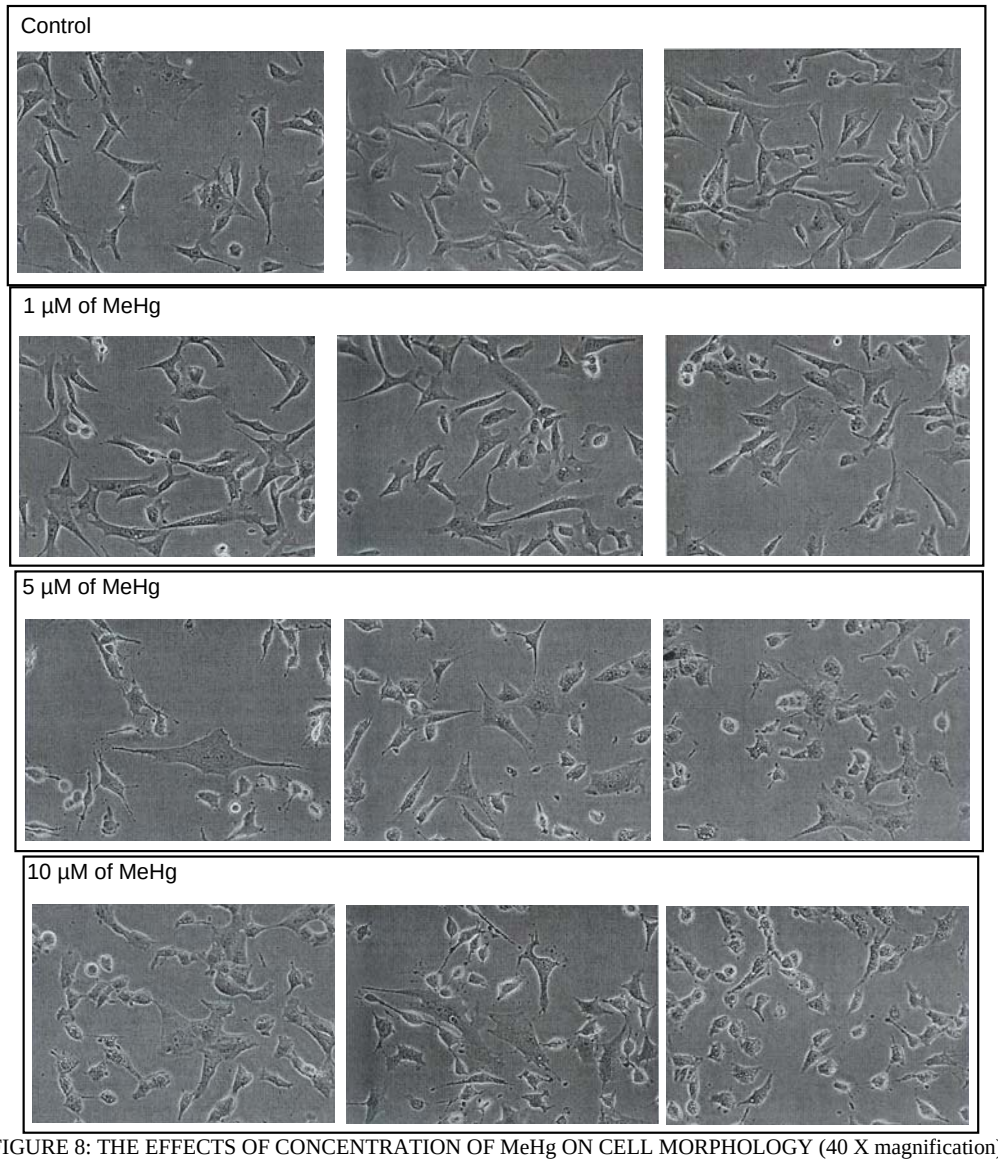

FIGURE 8: THE EFFECTS OF CONCENTRATION OF MeHg ON CELL MORPHOLOGY (40 X magnification)

	p-value	r value
LDH time response	1.6*10-4	.93
LDH dose response	7.7*10-8	.98
MTT time response	.049	-.88
MTT dose response	6.6*10-7	-.98

TABLE 2: A BEST FIT STATISTICAL ANALYSIS OF MEASURED DATA SETS

CONCLUSION

Using these various indicators of cell culture viability, a statistical test was run to determine a line or curve of best fit to encompass every data set measured, taking uncertainty into account (see table 2). This allowed the determination of statistically significant trials and their respective correlations.

If r>.90 is considered statistically significant best fit for a data set, then the MTT time-response is the only data set that does not fit this correlation. The LDH time-response is also on the borderline for statistical significance. This is due to the noticeable drop in r-value for time-response trials for both LDH and MTT. Both of these have larger uncertainties present, as well as more gradual, less uniform trends. This should serve as an important preliminary indicator that MeHg exposed to cells for a longer duration of time is not as detrimental as greater concentrations of MeHg present in the cell culture. This observation is confirmed by the p-values themselves—as r-values get closer to the absolute value of 1, p-values get closer and closer to 0. This independent confirmation adds special credence to the significance of this data set. It shows that as data sets get closer to an actual line or curve of best fit, they also show higher levels of statistical difference from the control.

Through such statistical analysis, it has been shown that higher concentrations and prolonged durations of exposure to MeHg result in membrane degradation, mitochondrial dysfunction, altered cell morphology, and overall cell death.

These conclusions generally support the hypothesis first presented in this experiment (*MeHg has an adverse [negative] effect on endothelial cells*), but with qualification: the duration of exposure to MeHg still only has borderline statistical significance to show adverse effects on cell culture.

Yet the verification of at least part of this hypothesis shows the beginning of a successful bridging of a gap present in MeHg toxicology literature. It is one of the first observations to show concrete cellular and molecular data to corroborate the connection between MeHg toxicity and vascular disease. Yet these data sets need further statistical scrutiny and corroboration with past research.

Applications of this study are manifold. Assays need to be carried out to pinpoint the exact biochemical processes that allow MeHg to lead to vascular cell dysfunction. Such information then needs to be compared to how MeHg is known to act in the body as a neurotoxin. Effects on other cell lines also need to be studied so that a more comprehensive understanding of MeHg effects can emerge. The poisoning of such heavy metal toxins is well-documented, and more biochemical understanding is necessary to effectively combat it.

The limitations for this study are also important to consider. The study needs more data of in vivo mercury toxicity over longer periods of time to continue to justify cellular exposure periods. This needs to be compared side-to-side with a representative human population with varying levels of observed blood MeHg concentration. In vivo concentrations need to correlate with morphology trials and in vitro statistics so that assay values can gain significance to real-life cardiovascular risk. The ultimate use of this study should expand known biochemical models of cardiovascular MeHg toxicity. A recent study has already shown that hyperoxia in the artery lining causes the activation and regulation of certain kinase and signal transduction-agents (Parinandi 2003). Since MeHg induces many reactive oxygen species in the body, redox-induced cardiovascular disease is one of the most promising paths for the

development of this toxicology model. To expand this study, the effects of MeHg on the development of these membrane agents need to be studied, especially those that promote cell apoptosis and necrosis. A cell-based model of chemical-specific toxicology will prove beneficial in further epidemiological studies.

In conclusion, this study is the first in the literature to explicitly link the epidemiological studies that relate cardiovascular disease and MeHg exposure with the adverse effects of MeHg at the endothelial cell level. This has profound implications on the nonconventional risk factors of cardiovascular disease.

This information is crucial for modern warfare. The high levels of MeHg production as a result of military industrial waste contribute in a large part to the anthropogenic production of Hg (Boening 2000). The biological relevancy of this once-thought neurotoxin is being rediscovered. Soldiers and medical personnel in levels of high MeHg toxicity need to be acutely aware of the toxin's effects on their soldiers in the long- and short-term, and this study opens a door to a better awareness of those cautions.

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